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PRINCIPAL INVESTIGATOR: Jennifer Brooks Ph.D.

CONTRACTING ORGANIZATION: New York University
New York, NY 10016

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Jennifer Brooks Ph.D.

E-Mail: brooksj@mskcc.org

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14. ABSTRACT
Promoter methylation of tumor-suppressor genes is a frequent and early event in breast carcinogenesis. Paired tumor tissue and serum samples from women with breast cancer show that promoter methylation is detectable in both sample types, with good concordance. This suggests the potential for these markers to be used for early breast cancer detection. This was a case-control study nested within the New York University Women's Health Study to assess the ability of promoter methylation in serum to detect pre-clinical disease. Cases were women with blood samples collected within the six months preceding diagnosis (n=50). Each case was matched to 2 healthy cancer-free controls and 1 cancer-free control with a history of benign breast disease. Promoter methylation of four cancer-related genes: RASSF1A, GSTP1, APC and RARβ2, was conducted using quantitative methylation specific PCR. Results of this analysis showed that the frequency of methylation was lower than expected among cases and higher than expected among controls (RASSF1A, 22.0%, 22.9% and 17.2% of cases, BBD controls and healthy controls respectively were methylated; GSTP1, 4%, 10.4% and 7.1% respectively; APC, 2.0%, 4.4% and 4.2% respectively and RARβ2, 6.7%, 2.3% and 1.1% respectively). Methylation status of the four genes included in this study was unable to distinguish between cases and either control group. This study highlights a methodological issues to be addressed before the evaluation of methylation markers as diagnostic biomarkers in prospective studies should continue, including the potential for false-positive and false-negative results when using a small amount of DNA template for QMSP

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Introduction

The promoter regions of some genes, in particular tumor suppressor genes, are frequently hypermethylated in cancer, but not normal cells. This methylation is thought to be an early event in carcinogenesis. Through necrosis and apoptosis, tumors release genomic DNA into the systemic circulation. Analysis of this DNA found in the serum/plasma of breast cancer cases, allows for the detection of promoter hypermethylation, with results showing good concordance with paired tumor tissue samples. We proposed to assess the potential of serum DNA hypermethylation markers as a tool for early detection of breast cancer. To date, no study has been conducted using serum collected prior to breast cancer diagnosis. Such a study can only be conducted using the resources of a large cohort with access to blood samples collected prospectively in healthy women, such as the NYU Women's Health Study (NYUWHS).

The NYUWHS enrolled 14, 274 women aged 35-65 between the years 1985 and 1991. Serum was collected from each participant and stored for future biochemical analyses.
At the time of the last complete round of follow-up, 1,006 cases of breast cancer had been diagnosed. This project is a nested case-control study within this cohort. Women for whom we have a blood sample collected within the 6 months preceding breast cancer diagnosis (n=113) will form the case group. For each case, controls will be selected and matched for age at, and date of, blood donation. The analysis of the promoter methylation status of a panel of six cancer-related genes (*RASSF1A, GSTP1, RARβ2*, *ERβ*, *DAPK* and *CDKN2A*) was proposed.

Body

Training Plan: At this stage all aspects of the Training Plan have been completed. In June 2009 I successfully defended my dissertation research on which this grant is based.

Work Plan:

Task 1: Case-control Selection

A total of 1,006 invasive breast cancer cases were diagnosed prior to 7/1/03, the start date of our latest complete follow-up. A total of 3,074 women with a history of benign breast disease have also been identified. Cases are women for whom we have a blood sample collected within the 6 months prior to breast cancer diagnosis (n=113). For each case, two sets of controls were selected. In the first set, two healthy controls were selected at random from women who were alive and free of any cancer and who had no history of BBD. In the second set, one control subject was randomly selected among healthy cancer-free women with a history of BBD. Controls were matched to cases for age and date of blood donation ± 6 months.

A series of selection criteria and priorities were created to facilitate the selection of appropriately matched controls. In an ideal match the control's age is within ±6 months of the case's age and the date of blood donation in the control is within ±6 months of the date of blood donation of the case. To be included in the "healthy" control group, subjects must have been free of benign breast disease at baseline and ANY cancer for the duration of the study to date. Those women in the "Benign Breast Disease" control group needed to be free of ANY cancer for the duration of the study.

The primary objective of this study is to determine whether the promoter methylation status of a panel of genes can be used for the early detection of breast cancer. This makes the cancer free status of the controls the most important selection criterion. To conduct the appropriate comparison between case and control methylation status, needed to meet the study objective, it is important to know that the control did not become a case later on in the study and therefore may have had undiagnosed, early stage breast cancer at the time of blood donation. This is especially true in the case of promoter hypermethylation given that it is believed that these changes occur early on in the development of the tumor.

When an ideal match was not possible, a series of relaxation criteria were established. The first relaxation was to extend the matching for date of blood donation to ±9 months while keeping all other criteria the same. If control selection was still not possible then the variation in date of blood donation was increased in 3 month increments up to ±18 months. At this point, if a

control was still not available, the variation in age was increased in 6 month increments, up to ±2 years.

In the first 50 case-control sets, of the 150 controls selected only 7 required the relaxation of selection criteria. For 4 controls the difference in dates of blood donation was extended to ±9 months, and for 3 controls to ±12 months.

Task 2: DNA Isolation

DNA was isolated from 1 ml aliquots of serum using the QIAamp DNA Blood Midi Kits (Qiagen, Valencia CA) as described by the manufacturer with a few minor modifications due to the expectation of small amounts of DNA being isolated. These changes have been extensively validated in Dr. Wirgin's laboratory where analysis of NYUWHS DNA has been conducted for the past five years. Samples from each case-control set were isolated in the same batch, on the same day and stored for the same length of time before DNA modification by sodium bisulfite treatment. Isolated DNA was stored in six 45µl aliquots at -80°C to eliminate any unnecessary freeze-thaw. Each aliquot is the amount required for the sodium bisulfite conversion assay and sufficient for the methylation analysis of two genes of interest and the reference gene.

Task 3: Method Optimization and DNA Methylation Analysis

DNA methylation analysis requires two basic steps. First the DNA must be chemically modified using sodium bisulfite, converting unmethylated cytosines to uracil while leaving methylated cytosines unchanged. This treatment leads to the generation of detectable methylation specific sequence variation. Once treated, DNA is amplified using fluorescence based, quantitative real-time PCR (QMSP) using the AB7300 (Applied Biosystems, Foster City CA). Optimization of the sodium bisulfite treatment method and QMSP analysis has been completed.

Sodium Bisulfite Conversion of DNA

In the original proposal sodium bisulfite conversion was to be carried out using the method by Herman et al (1). However, since the time of the original grant submission a number of kits became available for the sodium bisulfite treatment of DNA. After consultation with those in Dr.

Klein's laboratory, in which two different kits had been used, the Qiagen Epitect Bisulfite conversion kit (Qiagen, Valencia CA) was selected. Using the QIAGEN kit increased the speed with which the samples were analyzed. Kits were tested using standards of fully methylated and fully unmethylated DNA (Millipore, Billerica MA). Bisulfite conversion was conducted as described by the manufacturer. Modified DNA not analyzed immediately was stored at -20°C until further use.

Samples from each case-control set were treated in the same batch, on the same day and stored for the same length of time upon conversion. Standards for each PCR plate were also treated in the same batch as the samples for that plate. Usually, samples were analyzed on the same day of sodium bisulfite conversion to eliminate the effects of storage completely.

Analysis of DNA Methylation

Bisulfite treated DNA was amplified using QMSP. This method can attain a detection sensitivity of up to 1 in 10,000, compared to a sensitivity of 1 in 1000 for traditional methylation specific PCR (MSP) (2). Amplification was conducted using locus and methylation specific primers, flanking a sequence specific, 18-20bp, dual labeled, TaqMan® probe. Increased specificity is gained by the specificity of not only the forward and reverse primers, but the probes as well. Fluorescence was detected using the AB 7300.

Real-time PCR was carried out as described by Eads et al (2). Briefly, for each assay two sets of primers and probes were used. The first set of primers was designed to recognize the sequence of the methylated, bisulfite treated gene of interest. The second set, for *β-Actin* (*ACTB*), was run in parallel and used as a control to normalize for DNA input. Primer and probe sequences were obtained from previous publications and reported in the first progress report.

Standard curves using fully methylated DNA and probing for the genes of interest and for the control gene (*ACTB*), are included in each plate. This acts as a positive control and allows for the quantification of promoter methylation relative to a fully methylated control. It also controls for sample DNA input. Standard curves are generated from the same stock solution (3.3ng/µl) and can therefore also act as a control for plate-to-plate variability. Dilutions of methylated DNA are run from 1,000 copies (660 pg DNA/µl) down to 1 genome copy (0.66 pg DNA/µl). Standard

curves with high r-squared values and slopes close to -3.33 are the most accurate. Cases and their 3 controls (2 healthy, 1 BBD) are run on the same plate. This ensures that any differences in methylation seen between the 3 groups are not due to plate-to-plate variability or differences in DNA storage time.

To optimize assay efficiency with respect to the limited amount of sample DNA, two target genes were run for each sample on one plate (using one aliquot of isolated DNA). This was run along side the *ACTB* control and allows the same control to be used for both genes. This decreases the amount of sample DNA needed for *ACTB* control reactions overall.

Each assay also included universally unmethylated DNA as a negative methylation control. Unmethylated DNA is included as a negative quality control on each plate to reduce the probability of false positive sample results. Inclusion of this control monitors the specificity of the primers and probes for methylated sodium bisulfite treated sequences as well as the efficiency of the bisulfite treatment reaction itself. Incomplete sodium bisulfite conversion can generate false positive results where unmethylated DNA (i.e. the negative control) is amplified using methylation specific primers. The negative control should only be amplified by *ACTB*, whose primers and probe are not methylation specific. This indiscriminant amplification is what allows it be used to quantify the amount of DNA template in each sample. Several water blanks were also included on each plate.

Amplification Conditions

The final composition of the master mix consisted of 1X TaqMan® Universal PCR Master Mix No AmpErase®, 600nM of each primer (forward and reverse) and 200nM MGB probe, with a final reaction volume of 50µl. Amplification conditions were as follows: 10 minutes at 95°C and then 95°C for 15 seconds followed by 60°C for 1 minute, for 50 cycles.

Gene Selection

The gene panel for this study included *RASSF1A*, *GSTP1*, *RARβ2* and *APC*. These genes were selected for their known involvement in carcinogenesis and because they have been shown to be methylated in the tumor tissue and serum of women with confirmed breast cancer.

Results of Methylation Analysis

Methylation analysis was conducted for 50 cases and their matched controls (n=200). **Table 1** shows the frequency (95% CI) of methylation in cases and controls, as determined by amplification above the threshold (PMR>0). The methylation frequencies of the four genes analyzed were low among all three subject groups. Further, these frequencies were not able to distinguish between cases, controls with a history of BBD and controls without a history of BBD. Because no significant differences between the two control groups were observed, the control groups were combined and the analysis repeated. Again, no significant differences in the frequency of methylation were observed between cases and controls. Only for *RASSF1A* and *RARβ2* was the frequency higher (non-significantly) in cases than controls (*RASSF1A*: 22% of cases, 19% of controls; *RARβ2*: 6.7% of cases and 1.5% or controls). Among those women with a PMR>0, PMR values did not differ between cases and the combined control group for any gene (results not shown), though the interpretation of these comparisons was limited by the small number of subjects with a PMR>0 (Table 3). Overall, 31.8% of cases and 28.8% of controls (BBD and healthy controls combined) had methylation in at least one gene.

Methylation assays were shown to have good sensitivity in the standards (dilutions of fully methylated DNA), able to detect down to one genome copy. Standards were shown to have a high level of reproducibility between plates, as indicated by low inter-plate coefficients of variability (CV) for each gene: *RASSF1A*: 11%; *GSTP1*: 3%; *APC*: 2%; *RARβ2*: 1%. When repeat Ct values (the Ct value is the point at which amplification cross the detection threshold) for *ACTB* were compared between plates, they too were found to have a low CV of 3%. This level of variability did not differ between cases and either control group. Though the Ct values were highly reproducible between plates, the corresponding copy number was highly variable with a CV of 53.2% overall. This increase in variability is seen because a small difference in Ct translates into a large difference in copy number once the copy number is log transformed and made linear.

To further confirm that the amplification seen with QMSP was due to the presence of methylation and not an artifact of the QMSP procedure (due to low DNA input and high cycle number) or incomplete sodium bisulfite conversion, a subset of samples were selected (blinded

to case-control status) and bisulfite sequenced (n=12 for *RASSF1A* and n=7 for *GSTP1*). Because the PCR products of the QMSP reactions are less than 100bp, direct DNA sequencing was not possible and bacterial cloning was required. Samples were selected so that a cross-section of amplification threshold values would be used. This was done in an attempt to determine an appropriate cut-off point to be used for the classification of samples as being methylated.

Bacterial Cloning Procedure

DNA samples were sodium bisulfite treated (as described) and MSP was conducted using a final reaction volume of 25µl. This included 1 × PCR Buffer (Qiagen, Valencia CA), 200µM dNTPs, 60nM of each (forward and reverse) methylation specific primers (Applied Biosystems, Foster City CA) and 1 U Hotstart Taq Polymerase (Qiagen, Valencia CA). Conditions were as follows, 95°C for 15 minutes followed by 45 cycles of 94°C for 20 seconds, 57°C for 30 seconds and 72°C for 30 seconds, followed by a hold at 4°C. Product was visualized by 10% TBE polyacrylamide gel electrophoresis.

The bacterial cloning reaction was carried out using the TOPO® TA Cloning Kit for sequencing (Invitrogen, Carlsbad CA). Briefly, the vector ligation reaction was conducted directly after the completion of the MSP program. This reaction included 3µl of fresh PCR product, 1µl of salt solution, 1µl water and 1µl of TOPO® vector for a total volume of 6µl. Once combined the reaction was mixed gently and incubated for 5 minutes at room temperature. The reaction was then put on ice or stored at -20°C until use.

Transformation of One Shot® TOP10F' Competent Cells (Invitrogen, Carlsbad CA) was carried out using 3µl of ligation reaction and 1µl for the pUC19 control provided. DNA was added directly to a 50µl vial of cells and mixed gently by tapping. Reactions were then incubated on ice for 30 minutes followed by 30 seconds in a 42°C water bath and then back on ice. Using sterile techniques, 250µl of room temperature SOC medium was added to each vial. Reactions were then placed in a rotary shaker incubator on their side and incubated at 37°C for exactly 1 hour while shaking at 225 rpm. Following this, samples were plated on LB Agar plates containing 0.1 mM IPTG, 0.004% galactose in dimethylfluoride and 100µg/µl ampicillin. 100µl of

SOC medium was first added to each plate, followed by 80µl from each transformation vial (50µl for the pUC19 transformation control). Plates where then inverted and incubated overnight at 37°C.

The next morning four colonies (1 blue and 3 white) were selected from each plate and placed in culture tubes containing 2ml of LB medium with 100µg/µl ampicillin. Tubes were then put in the rotary shaker incubator overnight and incubated at 37°C while shaking at 225 rpm. After this, cultures were spun down and the medium removed. Plasmid DNA was isolated using the PureLink Quick Plasmid Mini-prep Kit Protocol (Invitrogen, Carlsbad CA) according to the manufactures instructions. Once isolated, DNA was stored at 4°C for immediate use or at -20°C for later use.

DNA Sequencing

Sequencing of isolated plasmid DNA was conducted by capillary gel electrophoresis (CEQ-8000) using a Dye Terminator Cycle Sequencing (DTCS) kit (Beckman Coulter, Fullerton CA). The sequencing reaction was prepared containing 3µl of plasmid DNA, 5pmol of the M13 Reverse plasmid sequencing primer (Invitrogen, Carlsbad CA) and 6µl of DTCS (Beckman Coulter, Fullerton CA). Reactions were run at 96°C for 20 seconds, 50°C for 20 seconds and 60°C for four minutes, for 40 cycles followed by a hold at 4°C. Samples were then ethanol precipitated and placed in a speed-vac for 15 minutes to dry down. Next, 40µl of sample loading solution (Beckman Coulter, Fullerton CA) was added and samples were capped and allowed to sit for 10 minutes, gently vortexed for 30 seconds and spun down for 10 seconds. Each sample was then transferred to CEQ plates and covered with mineral oil. Plates were run using the shorter LFR-c sequencing program.

Samples included in the analysis were selected so that a cross-section of amplification threshold values (Ct values) was used. The difference in Ct values between the sample gene of interest reaction (i.e. *RASSF1A*) and its *ACTB* reaction on the same plate was used. The greater the proportion of DNA copies that are amplified in the sample, the smaller the difference between Ct values. Selection was conducted in this manner also in an attempt to generate data that could be used to create a rule for Ct difference that could classify a sample as methylated.

This could give a more accurate indicator of methylation status, rather than using any amplification beyond the threshold as the criteria.

Samples that amplified for *RASSF1A* were selected for sequencing because this gene had the highest number of samples amplified beyond the threshold, suggesting the potential for the greatest proportion of false-positives. In contrast, a small group of samples that amplified for *GSTP1* (comparatively few samples amplified for this gene) were also sequenced. This procedure remains the gold standard for methylation analysis though it is limited by its high cost and low sample throughput.

Results showed that for *RASSF1A*, all but two of the samples that amplified during QMSP were methylated. In both instances the sample was from a control subject (1 healthy control and 1 BBD control). It is possible that these samples would have been found to be methylated had more colonies been selected for analysis. In contrast, bisulfite sequencing revealed that none of the samples selected for QMSP amplification for *GSTP1* were methylated. In this instance however, by chance (because the samples were selected while blinded to case-control status) all the samples included in the analysis were controls (3 healthy controls and 4 BBD controls).

These results suggest that in the case of *RASSF1A*, the results of the methylation analysis accurately determine the methylation status of the sample. However, in the case of *GSTP1*, this may not be true, since all of the samples selected for sequencing were found to be unmethylated. Further, the results did not provide an indication of a Ct difference that could be used for a more accurate means of methylation classification. This gives the first indication that QMSP may not perform well on samples containing only small amounts of DNA template. It also suggests that some of the amplification seen could be due to non-specific binding after a large number of PCR cycles. Sequencing also provided further confirmation that the samples were being completely converted during the sodium bisulfite treatment step.

DNA Quality and Quantity

The QMSP results showed that while the repeat measures of standards had low variability and good reproducibility, the same was not true for samples. The samples being used for this study are from the NYUWHS which was initiated in the mid-eighties. This means that the serum samples, from which the DNA is obtained, have been stored for approximately 20 years at -

80°C. It was hypothesized that one possible reason the samples and standards where behaving differently in the analysis was that the samples had been damaged at some point during their long-term storage.

To test this hypothesis fresh blood samples were collected from 6 healthy individuals. Serum was separated according to the protocol detailed by the NYUWHS (3) and stored at -80°C. DNA was isolated using the Qiagen method and stored in aliquots of 45µl at -80°C until time of analysis. Samples were then analyzed and compared to NYUWHS samples with respect to quantity and quality.

DNA quantity was determined by looking at the copy number obtained for *ACTB* using QMSP. DNA quality was assessed using a PCR based fragment assay as described by van Beers et al (4). For this experiment isolated DNA (10µl) from freshly collected normal samples and study sample DNA were amplified in a multiplex PCR reaction that included 4 sets of primers specific for fragment sizes of 100, 200, 300 and 400 bps for the *GAPDH* gene. If samples have a greater proportion of small fragments, the sample is considered to be more fragmented. Fragmentation can disrupt the detection of promoter methylation if it occurs at the primer/probe binding sites.

PCR reactions for this analysis included 1 × PCR Buffer (Qiagen, Valencia CA), 200µM dNTPs, 132nM of each primer (forward and reverse) (Applied Biosystems, Foster City CA) for each fragment size and 1 U Hotstart Taq Polymerase (Qiagen, Valencia CA). Samples were then run for 15 minutes at 95ºC and then 1 minute at 94ºC, 1 minute at 56ºC and 3 minutes at 72ºC for 40 cycles followed by 7 minutes at 72ºC. Reactions were then visualized on 10% TBE polyacrylamide gel electrophoresis.

ACTB was quantified in the fresh samples to determine the number of genome copies present in the samples. After five repeats, *ACTB* was not detectable in two of the samples, later experiments showed that DNA was however present. In those samples that DNA was detectable, there was a high level of variability - as was seen in the NYUWHS samples. Results of the fragment analysis (reported in the previous annual report) also showed that though the samples did look somewhat different, the NYUWHS samples were not more fragmented than the fresh DNA samples. These results suggest that the issue of sample

variability is not due to quality of the DNA in the NYUWHS samples but perhaps related to the small amount of sample available for analysis.

Through a series of experiments it was found that the bisulfite treatment procedure was efficient and not generating false-positive results. Further, the quality of the DNA obtained from the NYUWHS appears to be comparable to that obtained from freshly collected DNA. This leaves as the likely cause of the lower than expected frequency of methylation in cases to be the low amount of DNA input available for QMSP analysis.

Median (10^{th}, 90^{th} percentile) *ACTB* copy number/ml and ng/ml for the NYUWHS samples are shown in **Table 2**. The 90^{th} percentile enters in to the range of DNA that might be expected from these samples (~100ng/ml). Here, the amount of *ACTB* present in a sample is shown to have a great deal of individual variability, unable to distinguish between cases and controls.

To investigate the influence of DNA quantity on the results, eight newly diagnosed breast cancer cases were recruited from the NYUCI. This was done so that a greater volume of serum could be available for analysis and to focus on women with stage 2 disease or higher, in an attempt to ensure a higher concentration of circulating DNA.

Women were recruited and blood was drawn prior to any treatment (e.g. chemotherapy, tamoxifen) or surgery. Of these women three had incomplete pathology information because they have yet to schedule surgery (**Table 3**). Using the pathology information available, the average age at diagnosis was 49.6 years, which is only slightly younger than the NYUWHS women (average of diagnosis 52.0 years). All NYUCI cases were diagnosed with IDC and of these 4 were ER-negative and 2 were ER-positive, 2 were reported as over-expressing HER2.

NYUCI women were found to have a median of 3,718 *ACTB* copies per ml of serum. This amount is less than that seen in the NYUWHS samples (Table 3). Though, statistical comparisons are not possible due to the small number of subjects, it was observed that the 2 women who were reported as over-expressing *HER2* were among those with the highest *ACTB* copy number in their sample (Samples 7 and 8). These, and Sample 2 (also with a high *ACTB* copy number) were also diagnosed with stage 3 tumors. Samples 2 had a high *ACTB* copy number but pathology information was not available for that patient.

Methylation analysis was conducted for *APC*. This gene was selected because it was the only gene to show the expected relationship between cases and controls based on PMR (i.e. cases had higher PMRs than controls; Figure 6). None of the 8 women were shown to have methylation in the promoter region of *APC*. Reproducibility between plates was comparable to that of the NYUWHS samples with an inter-plate CV of 2.5% for *ACTB* Ct values. Further, as seen in the NYUWHS samples, the variability was greatly increased when CVs were calculated based on repeat measures of *ACTB* copy number (inter-plate CV=59.1%). This lack of reproducibility restricts the validity of this method to relative (within a plate) rather than absolute measures.

Task 4: Statistical Analysis and Manuscript Writing

Statistical analysis has been completed and the manuscript detailing the results of this study is in preparation. It is expected that this manuscript will be submitted for publication in October, 2009. The Statistical Analysis section of that manuscript is detailed below:

"Subject characteristics of cases were compared to those of each control group (controls with a history of BBD, and healthy controls) using conditional logistic regression to take into account the matching. An analysis was also conducted comparing cases to the two control groups combined. In addition, a multinomial unconditional logistic regression adjusting for age was conducted to simultaneously compare all three groups (Table 4).

Methylation was examined as a dichotomous variable (0/1) that was coded as methylated (given a value of 1) if there was any amplification above the threshold. The percent of fully methylated DNA (PMR) was also calculated by taking the amount of DNA found to be methylated for the gene of interest, divided by the amount *ACTB* present for that same sample, multiplied by 100. The use of this variable however, was limited by the small number of subjects with a PMR>0 (n=39 for *RASSF1A*, n=14 for *GSTP1*, n=5 for *RARβ2* and n=7 for *APC*). Because of this, methylation analysis was restricted to that coding methylation as a dichotomous variable. Pair-wise comparisons of the frequency of methylation were conducted using exact conditional logistic regression to take into account the low frequency of methylation and the matched design of the study. *ACTB* copy number and concentration were $\log_2$-transformed and compared between cases and each control group using conditional logistic regression. All statistical analyses were conducted using SAS 9.1 (SAS Institute Inc., Cary NC)."

Additional publications directly related to this grant include a review detailing the methodological issues that came up over the course of this research. This article is in press (see attached).

As part of my involvement with the New York University Women's Health Study I also had the opportunity to work on other projects that have or will lead to additional publications. I published a paper on DNA repair polymorphisms and breast cancer risk (see attached) and am currently working on a paper looking at NSAID use and breast cancer risk (in preparation).

Task 5: Thesis preparation and defense

I successfully defended my dissertation in June 2009 and my thesis has been accepted by the Graduate School of Arts and Science at New York University.

Key Research Accomplishments

- Completion of all Training Plan and Work Plan tasks.
- Conferring of my Doctoral degree
- Multiple publications (see Task 4)
- Post-doctoral position at Memorial Sloan-Kettering Cancer Center

Reportable Outcomes

See attached CV .

Grants Received as a result of this Award

NYU Cancer Institute Translational Research Pilot Study Grant (Title: Serum Epigenetic Markers for the Early Diagnosis of Breast Cancer, P.I: Dr. Anne Zeleniuch-Jacquotte)
Funding Period: 1 year (01/07-12/07)

Susan G. Komen For The Cure, Basic, Clinical and Translational Research Grant (Title: Serum Epigenetic Markers and the Early Detection of Breast Cancer, P.I: Dr. Anne Zeleniuch-Jacquotte).

Funding Period: 2 years (07/01/07 – 06/30/09)

The Komen grant funds support laboratory supplies and efforts for the mentor of this project and the study data manager. The NYU Pilot Study grant allows for the addition of the BBD control group.

Positions Received as a result of this Award

In July of 2009 I began a 2 year post-doctoral position at Memorial Sloan-Kettering Cancer Center in the department of Epidemiology and Biostatistics. I am fortunate to have the opportunity to work with Dr. Jonine Bernstein as my advisor.

Summary and Conclusions

This study was the first to analyze DNA methylation in pre-diagnostic serum samples, using a rigorous study design to ensure the selection of healthy, cancer-free controls from the population giving rise to cases, helping to ensure that cases and controls were comparable with respect to demographic and socio-economic characteristics and that controls did not have early, undetected cancer. This type of design is also important to gain knowledge on frequency of methylation in "healthy" individuals. Unfortunately, the estimates obtained from our study are likely to be overestimates because of the misclassification of unmethylated samples as methylated by the QMSP method. Studies of high risk asymptomatic women have found relatively frequent methylation of some tumor suppressor and other cancer-related genes (5-6). For example, a study including fine-needle aspiration biopsies from 55 healthy women detected promoter methylation of *RARβ2* (9%), *APC* (26%), *H-cadherin* (17%) and *RASSF1A* (37%) (6), but little data are available for other, average-risk, individuals. In addition, the factors that may influence these frequencies, such as age and lifestyle characteristics, need to be better defined, as well as the direction and extent of their impact.

In summary, this study highlights a number of methodological issues to be addressed before the evaluation of methylation markers as diagnostic biomarkers in prospective studies should continue. These issues include the need to ensure that there is adequate DNA template in each reaction allowing the analytical method to reach its optimal level of sensitivity. Prospective

cohort studies are needed to test the ability of these markers to detect pre-clinical disease. The sample volume required for QMSP to accurately measure DNA methylation may be prohibitive for most existing prospective studies. Methylation detection methods leading to accurate results with use of small DNA amount are needed.

To aid in this effort, it is suggested that, prior to undertaking a large study, QMSP results be validated by conducting bisulfite sequencing on a subset of samples, as well as examining the concentration of DNA found in study samples. In doing so, the accuracy of the results when used on small amounts of DNA template will be clarified and the absolute sensitivity of the method determined. In order for these markers to be used as a screening tool on a healthy population, normal methylation patterns and the factors that affect these changes, when and how they occur, will also need to be clarified. Additionally, identifying those factors that may influence methylation analysis, such as the method of sample collection and sample handling and storage procedures will also be important.

All training and work tasks have been completed as detailed in the Statement of Work.

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JENNIFER D. BROOKS, PHD
Research Scholar
Memorial Sloan-Kettering Cancer Center
Department of Epidemiology and Biostatistics
307 East 63rd Street, 3rd Floor
New York, NY 10021
T: (646) 735-8068 F: (646)735-0012
brooksj@mskcc.org

EDUCATION

Present **PhD Candidate**: New York University School of Medicine, Department of Environmental Medicine, Division of Epidemiology
Thesis Title: Promoter Methylation of Tumor Suppressor Genes Detected in Serum for the Early Detection of Breast Cancer.
Advisor: Dr. Anne Zeleniuch-Jacquotte

2003 **MS**: University of Toronto School of Medicine, Ontario Canada – Department of Nutritional Sciences
Thesis Title: Phytoestrogens as Modulators of Estrogen Metabolism.
Advisor: Dr. Lilian U Thompson

1998 **BS** (Honors, Dean's List) Biomedical Science: University of Guelph, Ontario Canada

CERTIFICATES AND TRAINING

2004-2008 NYU School of Medicine IBRA HIPAA and Human Subjects Training
2007 National Institute of Environmental Health Science Environmental Genome Project: NIEHS SNPs Workshop, Columbia University

HONORS AND AWARDS

2006-2009 Department of Defense Pre-doctoral Training Grant
2001-2003 University of Toronto Open Fellowship

CURRENT FUNDING

Department of Defense Pre-doctoral Training Grant (Title: DNA Hypermethylation Patterns Detected In Serum As A Tool For Early Breast Cancer Diagnosis, P.I: Jennifer Brooks)
Funding Period: 3 years (09/06-09/09)

NYU Cancer Institute Translational Research Pilot Study Grant (Title: Serum Epigenetic Markers for the Early Diagnosis of Breast Cancer, P.I: Dr. Anne Zeleniuch-Jacquotte)
Funding Period: 1 year (01/07-12/07)

Susan G. Komen For The Cure, Basic, Clinical and Translational Research Grant (Title: Serum Epigenetic Markers and the Early Detection of Breast Cancer, P.I: Dr. Anne Zeleniuch-Jacquotte)
Funding Period: 2 years (07/01/07-06/30/09)

PROFESSIONAL SOCIETIES

2005- American Association of Cancer Research (AACR): Molecular Epidemiology Group (MEG) and Women in Cancer Research Group (WICR)
2008- International Genetic Epidemiology Society (IGES)

TEACHING EXPERIENCE

2005-2006 Epidemiology, Biostatistics, and Preventive Medicine – NYU School of Medicine
Teaching Assistant/Seminar Leader – 30 Medical Students
Contact Time: 1hr/week for 6 weeks
Prep Time: 6 hours

2002 Selected Topics in Food Science – University of Toronto School of Medicine

Teaching Assistant – 40 Senior Undergraduate Students
Contact Time: 1hr/week for 12 weeks
Prep Time: 12 hours

POSTERS AND PRESENTATIONS

2008 (poster) Department of Defense Era of Hope Breast Cancer Meeting – Baltimore MD
2007 Annual meeting of the International Collaborative Group on Hormones and Cancer – Gavi, Italy (presentation)
2002 (poster) Federation of American Societies for Experimental Biology – New Orleans LA

PUBLICATIONS

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Jennifer Brooks, Wendy Ward, John Hilditch, Jacqui Lewis, Leslie Nickell, Evelyn Wong, Lilian Thompson. Supplementation with flaxseed alters estrogen metabolism in postmenopausal women to a greater extent than does supplementation with an equal amount of soy. Am J Clin Nutr. 2004 Feb;79(2):318-25.

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Jennifer Brooks, Roy E Shore, Anne Zeleniuch-Jacquotte, Diane Currie, Yelena Afanasyeva, Karen L Koenig, Alan A Arslan, Paolo Toniolo, Isaac Wirgin. Polymorphisms in *RAD51*, *XRCC2*

and *XRCC3* are not related to breast cancer risk. Cancer Epidemiol Biomarkers Prev. 2008 Apr;17:1016-19.

Reviews, Chapters and Editorials

Lilian U. Thompson, Jim Chen, Kah Poh Tan, **Jennifer Brooks**, John Hilditch, Paul Gross. Flaxseed, Lignans and Breast Cancer: An Update. Proceedings of the 59th Flax Institute of the United States, Fargo, North Dakota, March 21-23: 47-51. 2002.

Jennifer Brooks, Anne Zeleniuch-Jacquotte, Paul Cairns. DNA Methylation Analysis for the Early Detection of Breast Cancer (Review): **in preparation**

Supporting Data

Table 1: Frequency of Promoter Methylation by Case-Control Status[A, B]

	RASSF1A	*GSTP1*	*APC*	*RARβ2*	At least One Gene Methylated[C]
	Frequency % (n) 95% CI	**Frequency % (n) 95% CI**	**Frequency % (n) 95% CI**	**Frequency % (n) 95% CI**	**Frequency % (n) 95% CI**
Cases	22.0 (11/50) 10.5, 33.5	4.0 (2/50) -1.4, 9.4	2.0 (1/49) -1.9, 5.9	6.7 (3/45) -0.6, 14.0	31.8 (14/44) 18.0, 45.6
BBD Controls	22.9 (11/48) 11.0, 34.8	10.4 (5/48) 1.8, 19.0	4.4 (2/46) -1.5, 10.3	2.3 (1/43) -2.2, 6.8	38.4 (15/39) 24.5, 52.3
Healthy Controls	17.2 (17/99) 9.8, 24.6	7.1 (7/99) 2.0, 12.2	4.2 (4/96) 0.2, 8.2	1.1 (1/88) -1.1, 3.3	24.4 (21/86) 15.3, 33.5

[A]Methylation Frequencies (PMR>0) by case-control status, no statistical differences were found (Conditional logistic regression).

[B]7 subjects were excluded due to undetectable ACTB levels in at least one of the assays (4 BBD controls and 3 healthy controls). In the case of RARβ2 additional samples did not amplify in the first round of analysis and were not repeated due to the precious nature of the samples and the results of the completed genes indicating that the frequency of methylation did not differ between cases and controls.

[C]Includes those women who had methylation measurements for all four genes

Table 2: Median Copy Number and Concentration of *ACTB* by Case-Control Status

	Median ACTB Copy Number/ml (10^{th}, 90^{th} percentile)	**Median ACTB (ng/ml) (10^{th}, 90^{th} percentile)**
Healthy Controls	6,375 (1,075, 28,751)	21.04 (3.55, 94.88)
BBD Controls	5,404 (268, 45,002)	17.83 (0.88, 148.50)
Cases	5,978 (444, 23,823)	19.73 (1.46, 78.62)

However, all 7 of the subjects that were found to have undetectable *ACTB* were controls, and would be expected to have the lowest amount of DNA present in their sample. To address the issue of low DNA concentration, a supplementary study was conducted.

Table 3: NYUCI Subject Characteristics*

Subject ID	Age at Dx (years)	Stage	Grade	Histology	ER + (yes/no)	PR + (yes/no)	*HER2* Over-expression	*ACTB* Copy Number/ml
1	48	1	2	IDC	yes	yes	no	703.70
2	62	3	3	IDC, DCIS	no	no	no	5309.78
3	44	-	-	IDC	-	-	-	5947.50
4	35	2	3	IDC	no	no	no	931.83
5	43	1	3	IDC, DCIS	no	no	no	2496.33
6	-	-	-	-	-	-	-	876.67
7	57	3	-	IDC	yes	-	yes	3450.91
8	58	3	3	IDC and ILC	no	no	yes	11,457.25

*Missing data (-) is due to incomplete pathology reports because of the early stage of a subjects treatment. IDC; inter-ductal carcinoma, ILC; inter-lobular carcinoma, DCIS; ductal carcinoma *in situ*, ER+; estrogen receptor positive, ER-; estrogen receptor negative.

Table 4: Subject Characteristics

Variable	Cases (n=50)	BBD Control (n=50)	Healthy Control (n=100)
Age at Index Date (years)			
Median (10^{th}, 90^{th} percentile)	52.0 (40.5, 65.9)	51.5 (40.4, 66.0)	51.8 (40.4, 65.8)
Menopausal Status, n (%)			
Premenopausal	22 (44)	21 (42)	43 (43)
Postmenopausal	28 (56)	29 (58)	57 (57)
BMI (kg/m^2)			
Median (10^{th}, 90^{th} percentile)			
≤52	23.4 (20.4, 29.0)	21.8 (19.1, 27.3)	25.5 (19.8, 32.8)
>52	25.8 (22.7, 31.0)	23.2 (21.4, 28.3)	24.4 (21.5, 31.2)
Unknown	0	0	1
Height (cm)			
Median (10^{th}, 90^{th} percentile)	162.6 (154.9, 170.2)	162.6 (152.4, 170.2)	162.6 (154.9, 172.7)
Unknown	0	0	1
Ethnicity, n (%)			
Caucasian	37 (74.0)	37 (80.4)	74 (78.7)
Black	8 (16.0)	4 (8.7)	13 (13.8)
Other (incl. Hispanic and Asian)	5 (10.0)	5 (10.9)	7 (7.5)
Unknown	0	4	6
Family History, n (%)			
None	42 (84.0)	40 (80.0)	77 (77.0)
1 affected relative, >45 yrs	4 (8.0)	10 (20.0)	15 (15.0)
>1 affected relative, or 1 age <45 yrs	4 (8.0)	0 (0.0)	8 (8.0)
Age at First Term Pregnancy, n (%)			
<25	16 (32.0)	24 (48.0)	34 (34.0)
25-29	12 (24.0)	6 (12.0)	22 (22.0)
Nulliparous	12 (24.0)	15 (30.0)	33 (33.0)
30+	10 (20.0)	5 (10.0)	11 (11.0)
Oophorectomy, n (%)			
No	49 (98.0)	47 (94.0)	90 (90.9)
Yes	1 (2.0)	3 (6.0)	9 (9.1 #0)
Unknown	0	0	1
Smoking, n (%)			
Never	19 (45.2)	25 (53.2)	49 (51.6)
Current	8 (19.1)	9 (19.2)	19 (20.0)
Past	15 (35.7)	13 (27.6)	27 (28.4)
Unknown	8	3	5
OC Use, n (%)			
Never	21 (56.8)	30 (61.2)	53 (58.9)
Ever	16 (43.2)	19 (38.8)	37 (41.1)
HRT Use, n (%)			
Never	46 (92.0)	42 (84.0)	92 (92.0)
Ever	4 (8.0)	8 (16.0)	8 (8.0)

*No statistical differences between any case-control groups were observed (conditional logistic regression).

Null Results in Brief

Polymorphisms in *RAD51*, *XRCC2*, and *XRCC3* Are Not Related to Breast Cancer Risk

Jennifer Brooks,[1] Roy E. Shore,[1,2] Anne Zeleniuch-Jacquotte,[1,2] Diane Currie,[3] Yelena Afanasyeva,[1] Karen L. Koenig,[1,2] Alan A. Arslan,[2,4] Paolo Toniolo,[2,4] and Isaac Wirgin[2,3]

[1]Division of Epidemiology, Department of Environmental Medicine; [2]New York University Cancer Institute; [3]Molecular Toxicology and Carcinogenesis Program, Department of Environmental Medicine; and [4]Division of Epidemiology, Department of Obstetrics and Gynecology, New York University School of Medicine, New York, New York

Introduction

Highly penetrant, but rare, mutations in genes involved in double-strand break repair (i.e., *BRCA1* and *BRCA2*) are associated with a risk for breast cancer of 40% to 65% by age 70 years (1, 2). Polymorphisms in other double-strand break repair genes are thought to contribute to the risk for the disease, either independently or through modifying the risk associated with rare mutations.

This study focuses on polymorphisms in three genes involved in the homologous recombination of double-strand breaks: *RAD51* 5′ untranslated region 135 G>C (rs1801320), X-ray repair cross-complementing group 2 (*XRCC2*) Arg^{188}His (rs3218536), and *XRCC3* Thr^{241}Met (rs861539) in relation to breast cancer risk in the New York University Women's Health Study cohort.

Materials and Methods

The New York University Women's Health Study cohort collected questionnaires and blood samples from 14,274 healthy women ages 35 to 65 years in 1985 to 1991 (3). The current nested case-control study is matched for age and date at blood donation and includes incident cases of invasive breast cancer diagnosed before March 1998, with further methodologic details described by Shore et al. (4).

DNA was isolated using Qiagen QIAamp Blood Mini Kits (Qiagen, Inc.; ref. 4). Genotyping was done using PCR-RFLP methods described previously (ref. 4; see Appendix 1 for gene-specific PCR conditions and primer sequences). Blood clots and/or cell aggregates were available for 48% of the women. For the remaining women, serum specimens were used. Genotype results from clots/red cells and serum showed excellent concordance between repeated samples ($n = 73$) in pilot studies (97% for *RAD51* 135 G>C, 99% for *XRCC2* Arg^{188}His, and 98% for *XRCC3* Thr^{241}Met). Quality control duplicates showed 100% concordance for all three polymorphisms.

Statistical Methods. Deviation from Hardy-Weinberg equilibrium was assessed in controls using the χ^2 goodness-of-fit test. The relationship between genotype and breast cancer risk was evaluated using conditional logistic regression and the additive coding model. The dominant model was also assessed for *RAD51* and *XRCC2* because of the small number of individuals with the homozygous variant genotype. Tests for interaction between genotype and ethnicity, family history, body mass index, and smoking were planned a priori.

Given our sample size (612 cases and 612 controls) and the allelic frequencies in our population, we had sufficient power (99% for *RAD51* 135 G>C, 99% for *XRCC2* Arg^{188}His, and 88% for *XRCC3* Thr^{241}Met) to detect associations of the magnitude observed by Kadouri et al. (5) for *RAD51* 135 G>C and Kuschel et al. (6) for *XRCC2* Arg^{188}His and *XRCC3* Thr^{241}Met.

Results

Genotype frequencies did not deviate from Hardy-Weinberg equilibrium ($P > 0.5$). Variant allele frequencies were comparable with those previously reported for populations of Caucasians of European descent for *XRCC2* Arg^{188}His (8%; refs. 6-9) and *XRCC3* Thr^{241}Met (36%; refs. 8-14), but the variant allele frequency for *RAD51* 135 G>C of 9% was somewhat lower than previous reports (5, 6, 9).

Table 1 describes study subject characteristics. As expected, significant differences in body mass index and parity/age at first full-term pregnancy were observed between cases and controls. However, these variables were not associated with genotype. Ethnicity was significantly associated with breast cancer risk and genotype. Asian and Hispanic women had a lower

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Requests for reprints: Anne Zeleniuch-Jacquotte, New York University School of Medicine, 650 First Avenue, 5th Floor, New York, NY 10016-3240. Phone: 212-263-6512; Fax: 212-263-8570. E-mail: anne.jacquotte@med.nyu.edu

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risk for breast cancer than non-Jewish White women (odds ratio, 0.49; 95% confidence interval, 0.29-0.81); this association is as expected (15). Ethnicity was significantly related to genotype for *RAD51* GC/CC ($P < 0.0001$) and *XRCC3* CT/TT ($P < 0.0001$) genotypes. Among Black women, 37.4% had at least one copy of the *RAD51* 135 G>C variant allele (non-Jewish White, 15.9%; Jewish White, 9.6%; others, 17.3%). The *XRCC3* $Thr^{241}Met$ variant was most common (67.3%) among Jewish White women (non-Jewish White, 60.3%; Black, 38.4%; others, 40.8%). *XRCC2* $Arg^{188}His$ variant was not significantly related to ethnicity.

Unadjusted and ethnicity-adjusted odds ratios and 95% confidence intervals are presented in Table 2. Although ethnicity was found to be related to genotype and risk, adjusting for ethnicity altered the odds ratios only slightly. In this population, none of the polymorphisms was found to influence breast cancer risk. The sum of variant alleles was also not related to risk (data not shown). Similar results were obtained when the analysis was restricted to Caucasians (data not shown). No significant interaction was found between genotype and ethnicity, body mass index, smoking, parity, or family history.

Discussion

Genetic instability acquired through inefficient double-strand break repair is believed to be a component of breast cancer susceptibility. RAD51 plays a central role in homologous recombination, through direct interaction with XRCC2, XRCC3, BRCA1, BRCA2, etc., to form a complex essential for the repair of double-strand breaks and DNA cross-links (especially XRCC2 and XRCC3) and for the maintenance of chromosome stability (16).

Studies have suggested that *RAD51* 135 G>C modifies the breast cancer risk of women with a family history of breast cancer (17, 18) or carriers of *BRCA2* mutations (5, 18-21). However, results have been inconsistent (22-24). Studies of non–*BRCA2* mutation carriers or women without a family history have found no association between *RAD51* 135 G>C and breast cancer risk (5, 6).

Table 1. Characteristics of cases and controls

Variables	Cases (n = 612)	Controls (n = 612)	Odds ratio (95% confidence interval)*	P*
Age at diagnosis (y)				
Median (25th, 75th percentile)	60.3 (51.8, 66.6)	60.3 (51.8, 66.6)	Matched	
Body mass index (kg/m^2)[†,‡]				
Median (25th, 75th percentile)				
Age ≤52 y	22.8 (20.9, 25.4)	23.1 (21.4, 25.0)	0.56 (0.11-2.75)	0.47
Age >52 y	25.2 (22.5, 28.4)	24.2 (22.0, 27.6)	2.20 (1.00-4.82)	0.05
Height (cm)				
Median (25th, 75th percentile)	163 (157, 168)	163 (157, 168)	1.00 (0.99-1.02)	0.72
Ethnicity, n (%)				
Caucasian				
Non-Jewish	222 (39.7)	202 (36.9)	1.00	0.02
Jewish	254 (45.4)	232 (42.4)	1.02 (0.77-1.35)	
Black	50 (8.9)	59 (10.8)	0.77 (0.49-1.21)	
Others (including Hispanic and Asian)	33 (5.9)	54 (9.9)	0.49 (0.29-0.81)	
Unknown	53	65		
Family history, n (%)				
None	468 (76.5)	475 (77.6)	1.00	0.31[§]
1 affected relative, >45 y	76 (12.4)	85 (13.9)	0.91 (0.66-1.27)	
1 affected relative, age unknown	15 (2.5)	12 (2.0)	1.24 (0.58-2.65)	
>1 affected relative or 1 age <45 y	53 (8.7)	40 (6.5)	1.33 (0.87-2.04)	
Age at menarche (y), n (%)				
<13	309 (50.5)	286 (46.7)	1.00	
≥13	303 (49.5)	326 (53.3)	0.87 (0.70-1.08)	0.20
Number of pregnancies, n (%)				
Nulliparous	201 (37.2)	180 (32.1)	1.00	0.19[§]
1	62 (11.5)	81 (14.5)	0.73 (0.48-1.10)	
2	153 (28.3)	173 (30.9)	0.77 (0.56-1.06)	
≥3	125 (23.1)	126 (22.5)	0.82 (0.58-1.15)	
Unknown	71	52		
Age at first term pregnancy (y), n (%)				
<25	142 (23.2)	183 (29.9)	1.00	0.0002[§]
25-29	168 (27.5)	180 (29.4)	1.21 (0.88-1.66)	
Nulliparous	201 (32.8)	180 (29.4)	1.47 (1.08-1.99)	
>30	101 (16.5)	69 (11.3)	1.96 (1.32-2.89)	
Smoking status, n (%)				
Never	253 (47.7)	253 (48.8)	1.00	0.35
Ever	278 (52.4)	265 (51.2)	0.99 (0.76-1.29)	
Unknown	81	94		

*Odds ratios and P values are for conditional univariate regression analysis.
† Using ln of body mass index (at baseline) as a continuous variable.
‡ A division at the age of 52 y was decided upon a priori as a surrogate for menopausal status.
§ P for trend using ordered categories shown in this table.

Table 2. DNA repair polymorphisms and breast cancer risk

Genotype*	Cases	Controls	Unadjusted		Ethnicity adjusted	
	n (%)	*n* (%)	Odds ratio (95% confidence interval)	*P*	Odds ratio (95% confidence interval)	*P*
Rad51 (*n* = 1,222) †						
GG	516 (84.5)	513 (84.0)	1.00	0.67 ‡	1.00	0.91 ‡
GC	88 (14.4)	88 (14.4)	0.99 (0.72-1.36)		1.05 (0.76-1.45)	
CC	7 (1.1)	10 (1.6)	0.67 (0.24-1.87)		0.68 (0.24-1.94)	
GG vs GC/CC			1.04 (0.76-1.41)	0.82	1.02 (0.74-1.39)	0.92
XRCC2 (*n* = 1,204) †						
GG	515 (85.5)	519 (86.2)	1.00	0.82 ‡	1.00	0.77 ‡
GA	83 (13.8)	78 (13.0)	1.07 (0.77-1.50)		1.08 (0.77-1.52)	
AA	4 (0.7)	5 (0.8)	0.81 (0.22-3.01)		0.83 (0.22-3.12)	
GG vs GA/AA			1.06 (0.76-1.47)	0.74	1.07 (0.77-1.48)	0.71
XRCC3 (*n* = 1,222) †						
CC	254 (41.6)	249 (40.8)	1.00	0.47 ‡	1.00	0.77 ‡
CT	259 (42.4)	286 (46.8)	0.88 (0.68-1.13)		0.83 (0.64-1.08)	
TT	98 (16.0)	76 (12.4)	1.28 (0.89-1.83)		1.20 (0.83-1.72)	

*Using the χ^2 test, no significant difference in genotype frequencies was observed between cases and controls.
† Matched pairs were excluded if either member of the pair could not be definitively genotyped.
‡ *P* for trend.

Results for *XRCC2* Arg^{188}His have been similarly mixed (6-8, 23). It is thought that this polymorphism has only a small effect on gene activity (7), although it may modify risk in those with low levels of plasma α-carotene (25) or plasma folate (26).

XRCC3 Thr^{241}Met has been found to be associated with increased DNA adducts (27), chromosomal deletions (28), and sensitivity to ionizing radiation and cross-linking agents (29, 30). Some (6, 17, 31) but not all (10, 23, 25, 32, 33) studies have found *XRCC3* Thr^{241}Met to be related to an increased risk for breast cancer. Pooled analyses and meta-analyses show a small but significant increase in risk (8, 14, 22, 34).

Disruption of double-strand break repair is thought to contribute to carcinogenesis through the accumulation of genetic errors and genetic instability (35). However, in this study, the *RAD51*, *XRCC2*, and *XRCC3* variants were found not to be associated with breast cancer risk. Unlike other reports, no relationship was found between *RAD51* 135 G>C and family history of breast cancer, perhaps because the participants in the study were not selected for having a family history of disease or being *BRCA1/2* mutation carriers.

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Appendix A. Gene-Specific PCR Conditions and Primer Sequences

Cycling conditions		
Rad51	95°C for 5 min	
	95°C for 30 s 65°C for 30 s 72°C for 60 s	33 cycles for red cells/clots, 37 cycles for serum
	72°C for 7 min	
XRCC2/XRCC3	95°C for 5 min	
	95°C for 30 s 65°C for 30 s 72°C for 60 s	30 cycles for red cells 32 cycles for clots 37 cycles for serum
	72°C for 7 min	
Primer pairs and restriction enzymes		
Rad51	TGGGAACTGCAACTCATCTGG GCGCTCCTCTCTCCAGCAG BstN1 (60°C for 2 h)	
XRCC2	GATTTTGGATAGCCTGTCA AGAATCATCTTGTTTGGAG SexA1 (37°C for 3 h)	
XRCC3	ATGGCTCGCCTGGTGGTCA CATCCTGGCTAAAAATACG NlaIII (37°C for 2 h)	

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REVIEW ARTICLE

Promoter methylation and the detection of breast cancer

Jennifer Brooks · Paul Cairns · Anne Zeleniuch-Jacquotte

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Abstract Mammographic screening has been shown to reduce breast cancer mortality in women over the age of 50 years, and to a lesser extent in younger women. The sensitivity of mammography, however, is reduced in some groups of women. There remains a need for a minimally invasive, cost-effective procedure that could be used along side mammography to improve screening sensitivity. Silencing of tumor suppressor genes through promoter hypermethylation is known to be a frequent and early event in carcinogenesis. Further, changes in methylation patterns observed in tumors are also detectable in the circulation of women with breast cancer. This makes these alterations candidate markers for early tumor detection. In this paper, we review the current literature on promoter hypermethylation changes and breast cancer and discuss issues that remain to be addressed in order for the potential of these markers to augment the sensitivity of screening mammography. In general, studies in well-defined populations, including appropriate controls and larger numbers are needed. Further, focus on the optimization of methods of methylation detection in small amounts of DNA is needed.

J. Brooks · A. Zeleniuch-Jacquotte
Division of Epidemiology, Department of Environmental Medicine, New York University School of Medicine, 650 First Avenue, 5th Floor, New York, NY 10016-3240, USA

P. Cairns
Department of Surgical Oncology, Fox Case Cancer Center, Philadelphia, PA 19111, USA

J. Brooks (✉)
307 East 63rd St. 3rd Floor, New York, NY 10065, USA
e-mail: brooksj@nyumc.org

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In the United States, over 12% of women born today can expect to develop breast cancer in their lifetime [1]. Women who are diagnosed at an early stage of disease have a better prognosis and require less severe treatment regimens than those diagnosed at an advanced stage [2]. Regular mammograms have been found to reduce breast cancer mortality in women over 50 years old and to a lesser extent in younger women [3–5], leading to the current recommendations that women at average risk should receive mammograms every 1 or 2 years, beginning at age 40 [6]. Indeed, in the United States, the majority of women over the age of 40 undergo mammographic screening [6, 7]. Suspicious mammographic findings lead to further testing that may include other imaging techniques such as magnetic resonance (MRI) and/or ultrasound, but ultimately the diagnosis is established by a biopsy, in particular to differentiate malignant from benign tumors. Although this well-established screening approach has led to a reduction in breast cancer mortality, it has a number of limitations pointing to the need for additional, complementary modalities which we briefly review in the following sections.

Need for complementary breast screening modalities

The sensitivity of screening mammography varies from about 68 to 93% [8], due to variations in practitioners' experience and skill [9] and patient characteristics. Specifically, mammography is up to 50% less sensitive in

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women who are young, Asian, on HRT, and/or have dense breasts [10–13]. It is also less sensitive for the detection of invasive lobular carcinomas (ILC) and small or diffuse tumors [9, 14, 15].

Specificity is an issue as well, with one in two women who receive yearly mammograms expected to have at least one false-positive result in her lifetime, leading to unnecessary biopsies and anxiety [16, 17]. Another concern is that a lifetime of yearly mammographic screening may eventually lead to a level of radiation exposure that increases breast cancer risk [18].

In light of these limitations, a minimally invasive screening test administered at the time of mammography, or prior to biopsy in the case of a suspicious mammogram, that would lead to overall greater sensitivity and specificity could have important public health value. To be maximally effective, this procedure should be able to provide information where mammography has deficiencies. That is, it should have the ability to distinguish between benign and malignant tumors and improve the sensitivity of detection for lobular carcinomas. It should also improve the sensitivity of detection in women with high breast density.

DNA methylation in cancer

Cancer initiation and progression is driven by the accumulation of inherited or acquired DNA mutations. These alterations may be genetic or epigenetic in nature [19]. Epigenetic modifications are changes in DNA structure that do not involve sequence changes but are stably inherited from cell to cell. These include DNA methylation, histone modifications (phosphorylation, acetylation, methylation), and microRNAs. Though these modifications may all show potential for early detection of cancer, this review focuses exclusively on DNA methylation.

Methylation of cytosine located 5′ to a guanosine can occur across the genome, but most notably within 0.5–4 kb CpG dinucleotide-rich regions, known as CpG islands [20–22]. Under normal conditions, the vast majority of CpG sites in the genome are methylated, with the exception of CpG islands located 5′ to the promoter and exon 1 of more than 50% of genes [22]. Methylation of gene promoter CpG islands is tightly linked to histone modifications and chromosome remodeling mechanisms that lead to gene silencing [23]. This method of controlling gene expression is widely used throughout the healthy genome. It is involved in the regulation of tissue- and time-specific gene expression (during differentiation and development), X chromosome inactivation in women, establishment and maintenance of imprinted genes, and the silencing of transposable elements [20].

The disruption of normal methylation patterns has been found to be an important event in carcinogenesis. In general, a shift to local promoter CpG island hypermethylation is seen within the context of an overall loss of methylation (hypomethylation). While global hypomethylation is thought to play a role in carcinogenesis primarily by increasing genetic instability, local hypermethylation alters gene expression [19]. Silencing of tumor suppressor genes through promoter hypermethylation is known to be a common event in carcinogenesis, thought to provide a selective growth advantage to tumor cells and contributing to the overall genetic instability of the tumor. This hypermethylation appears to be an early event in carcinogenesis [21, 24, 25], and occurs at least as frequently as genetic mutations in somatic cells so that hundreds of genes may be inactivated by DNA methylation in a single cancer [23, 26]. A large number of studies of breast cancer tissue have been conducted showing the frequent methylation of genes involved in cell cycle regulation: $p16^{INK4A}$, $p14^{ARF}$, *p15*, *CCDN2*, *DAPK*; DNA repair: *MGMT*, *hMLH1*; transformation: *GSTP1*; signal transduction: *RARβ2*, *APC*, *ERβ*; and adhesion and metastasis: *CDH1*, *CDH13*. The high frequency with which these alterations occur in cancer makes them potentially useful markers of disease.

Methylation markers in circulating DNA

Mandel and Metais [27] first discovered cell-free nucleic acids in the general circulation in the late 1940s. DNA is released into the circulation in healthy individuals and to a greater extent during pregnancy (from the placenta), trauma and after organ transplantation [28]. DNA is also present in the circulation of people with cancer [29] and in these individuals it has been estimated that as much as 93% of the total circulating DNA is derived from the tumor [30]. The mechanism of DNA release into circulation is poorly understood, but it is believed that DNA is released during tumor necrosis and apoptosis [30]. Because circulating levels of DNA are highly variable and are not limited to individuals with cancer, DNA concentration alone makes a poor cancer diagnostic tool; it can, however, be a source of biomarkers.

It was first recognized that tumors were the origin of some circulating DNA during the mid-nineties when it was found that it was possible to detect cancer-associated mutations (*N-ras*, *k-ras*) [28], microsatellite instability and loss of heterozygosity (LOH) identical to that seen in the tumor [31]. DNA alterations, including both mutations and epigenetic modifications, have also been detected in patients with small and in situ lesions. This suggests that tumors are releasing DNA early in the disease process, even before they become invasive [23], and therefore that

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circulating DNA may be a source of markers for tumor detection.

A number of studies illustrate the potential for the use of methylation markers in the early detection of a variety of cancers including prostate [32, 33], bladder [34, 35], gastric [36], renal [37], ovarian [38], colorectal [39], cervical [40], lung [41–43], liver [44], and breast [32–34, 37, 38, 45]. Studies in lung cancer have found that aberrant DNA methylation is detectable as early as 3 years prior to diagnosis in the sputum of subjects exposed to carcinogens (uranium miners and smokers) [42]. In a study of hepatocellular carcinoma, Santella et al. [44] detected changes in serum methylation patterns of *RASSF1A*, *p16*, and *p15* (using DNA from 200 µl of serum) as much as 9 years prior to diagnosis.

DNA methylation and breast cancer: results from tumor tissue

Table 1 shows that some genes are frequently methylated in tumor tissue DNA obtained from women who have been diagnosed with breast cancer [24, 46–51]. These studies have been conducted using a wide range of gene panels, though there is overlap among them. The sensitivity of detection, which ranges from 60 to 100% depends on the gene panel and the histological type of breast cancer. Studies have also shown that aberrant methylation events occur early on in breast cancer development, and are detectable in tissue from in situ carcinomas (both lobular and ductal) [24, 52] and early stage breast cancer (stages 0 and I) [49, 53].

To be an effective marker, gene panels in methylation studies must include genes that are methylated specifically in cancer and not in normal tissue. Fackler et al. [24] looked at the promoter methylation of a panel of genes (*RASSF1A*, *CCND2*, *TWIST*, *HIN1*) in samples obtained from invasive carcinoma and normal tissue adjacent to the tumor. They found that promoter methylation was more frequently detected in tumor than in normal tissue, though low levels of methylation were detected in normal control samples. In another study [54], normal tissue samples from the quadrant opposite of the primary tumor ($n = 12$) showed methylation of each of the 23 genes examined, except for *CDKN2*. ROC curve analysis showed that a panel of 4 of these 23 genes (*CCND2*, *RASSF1A*, *APC*, and *HIN1*) was able to distinguish between invasive carcinomas ($n = 66$), fibroadenomas ($n = 31$) and normal tissue ($n = 12$) [54]. Despite the fact that low levels of methylation were found in normal tissue, ROC curve analysis was still able to distinguish between normal samples and those with cancer.

A possible explanation for the methylation seen in some controls included in these studies is that, although not cancerous, the tissue samples may actually not have been 'normal'. Alternatively, there may be a threshold for methylation to affect gene expression and lead to a growth advantage. A better understanding of methylation frequencies detectable in 'normal' tissue is needed and will aid in the selection of the appropriate source for normal tissue (i.e., proximal to the tumor, from the contralateral breast) to use as control in comparative studies.

Distinguishing between benign and malignant disease

Benign breast diseases (BBD) are a diverse group of lesions which are poorly understood. BBD is an established risk factor for breast cancer, although the magnitude of the subsequent breast cancer risk remains controversial [55], partly due to the heterogeneity among types of BBD. Irrespective of its preneoplastic potential, it is important to consider BBD when assessing a potential breast cancer screening tool. BBD is extremely common; for example, postmortem studies have estimated that one in two women develops fibrocystic disease and one in five fibroadenoma during her lifetime [56]. Mammography is not always able to distinguish between cancer and BBD; this often requires a biopsy. A serum detection marker able to distinguish between benign and malignant breast tumors could reduce the number of breast biopsies and therefore have important public health value.

Studies testing the ability of promoter methylation profiles to distinguish between benign and malignant disease have led to mixed results [47, 48, 54, 57–59]. A study including women with invasive ($n = 24$), in situ ($n = 10$), and benign disease ($n = 8$), as well as healthy controls ($n = 20$) found that promoter methylation of three genes (*APC*, *RASSF1A*, *DAPK*) was detectable in DNA obtained from both in situ lesions and invasive samples at all tumor stages. No methylation, however, was found in the controls or benign breast disease patients [53]. In a different study, using a panel of genes including, *BRCA1*, $p16^{INK4A}$, *ESR1*, *GSTP1*, *TRβ1*, *RARβ2*, *HIC1*, *APC*, *CCND2*, and *CDH1*, it was found that fibroadenomas ($n = 10$) had patterns of methylation that were similar to that seen in breast cancer cases ($n = 54$), with the exception of *CDH1*, which was more frequently methylated in cases than in benign breast disease. *CDH1* is known to be involved in cell adhesion and tumor progression [60], so it may have high specificity for invasive disease. Eighty-five percent of breast cancers and 70% of fibroadenomas had methylation of at least one of the genes in the panel with half of the cases having methylation in three or more genes [47].

In a study using breast tissue samples obtained by FNA biopsy from women with benign and malignant tumors ($n = 27$) and unaffected women ($n = 55$), a panel containing *RASSF1A*, *RARβ2*, *APC*, and *CCND2* found that

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Table 1 Characteristics of selected studies of promoter methylation detected in tissue

Reference	Type of tumor (number of cases)	Genes	Methylation frequency	Coverage[a]
Fackler et al. [46][b]	LCIS	RASSF1A	62%	69%
	($n = 13$)	HIN-1	46%	
		RARβ	46%	
		CCND2	23%	
		TWIST	23%	
	ILC	RASSF1A	84%	100%
	($n = 19$)	HIN-1	79%	
		RARβ	21%	
		CCND2	32%	
		TWIST	16%	
	DCIS	RASSF1A	75%	95%
	($n = 44$)	HIN-1	68%	
		RARβ	48%	
		CCND2	32%	
		TWIST	27%	
	IDC	RASSF1A	70%	100%
	($n = 27$)	HIN-1	60%	
		RARβ	41%	
		CCND2	52%	
		TWIST	56%	
Fackler et al. [24][c]	Cases	RASSF1A	68%	84%
	($n = 19$)	TWIST	67%	
	($n = 21$)	CCND2	57%	
	($n = 21$)	HIN-1	57%	
	($n = 21$)			
	Controls	RASSF1A	7%	
	($n = 28$)	TWIST	6%	
	($n = 18$)	CCND2	14%	
	($n = 14$)	HIN-1	7%	
	($n = 16$)			
Parella et al. [47][b]	Cases	BRCA1	17%	85%
	($n = 54$)	P16	18%	
	(44 IDC, 10 ILC)	ESR1	46%	
		GSTP1	13%	
		TRβ2	28%	
		RARβ2	20%	
		HIC1	48%	
		APC	28%	
		CCND2	11%	
		CDH1	39%	
	BBD	BRCA1	20%	
	$n = 10$	P16	20%	
		ESR1	40%	
		GSTP1	0	
		TRβ2	0	
		RARβ2	0	
		HIC1	30%	
		APC	10%	
		CCND2	0	
		CDH1	0	

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Table 1 continued

Reference	Type of tumor (number of cases)	Genes	Methylation frequency	Coverage[a]
Jeronimo et al. [48][b]	Cases ($n = 27$)	CDH1	66%	88%
		GSTP1	58%	
		BRCA1	40%	
		RARβ2	34%	
Tao et al. [49][c]	Cases ($n = 803$)	CDH1	20%	60%
		p16	26%	
		RARβ2	28%	
Shinozaki et al. [50][b]	Cases ($n = 151$)	RASSF1A	81%	Not calculated (at least 81%)
		APC	49%	
		TWIST	48%	
		CDH1	53%	
		GSTP1	21%	
		RARβ2	24%	
	Controls ($n = 10$)	All genes	0	
Li et al. [51][b]	Cases ($n = 193$)	RARβ2	26%	Not calculated (at least 84%)
		CDH1	80%	
		ESR1	84%	
		BRCA1	41%	
		CCND2	11%	
		p16	14%	
		TWIST	59%	

[a] Coverage, percentage of cases having methylation of at least one gene in the given gene panel (i.e., coverage of 100% means that all samples had methylation of at least one gene in the study's panel); *LCIS* lobular carcinoma in situ; *ILC* invasive lobular carcinoma; *DCIS* ductal carcinoma in situ; *IDC* invasive ductal carcinoma; *BBD* benign breast disease

[b] Methylation analysis was conducted using methylation-specific PCR (MSP)

[c] Methylation analysis was conducted using quantitative real-time methylation-specific PCR (QMSP)

DNA from BBD lesions had an intermediate level of methylation, when compared to breast cancer cases and healthy controls [58]. Promoter methylation (especially of *APC* and *RASSF1A*) was also found to be more frequent in healthy women predicted to have a high risk of breast cancer (using the GAIL model), than those predicted to have a low/intermediate risk. Further, using three of these same genes (*RARβ2*, *RASSF1A*, and *CCND2*), in a study of 36 BBD, 21 in situ carcinoma and 45 invasive carcinoma, Pu et al. [59] found there was an increase in the frequency of promoter hypermethylation from benign (42% had methylation of at least one of the three genes) to in situ carcinoma (76%) and invasive carcinoma (96%).

These have been small studies using variable gene panels on a wide range of benign conditions that are usually not specified. Further, the age of the subjects participating in these studies was not reported. This could have important implications on the interpretation of the results, since the methylation of tumor suppressor genes in benign breast epithelium has been shown to increase with age [61]. Additional research is needed to assess the ability of promoter methylation analysis to distinguish malignant from benign conditions, with distinctions made between the type of BBD being studied and control for potential confounders such as age.

Detecting lobular carcinomas

Invasive lobular carcinoma (ILC) accounts for approximately 14% of all invasive breast cancers [62] and its incidence is rising [63]. MRI appears to be a more efficient tool to detect ILC than mammography [63], which besides being inefficient in the detection of ILC is also unable to distinguish between ILC and invasive ductal carcinoma (IDC) [64]. Promoter methylation may prove to be a useful tool to improve the detection of ILC.

Fackler et al. [46] and Pu et al. [59] conducted studies comparing the methylation patterns in ILC and IDC. They found that, overall, the two histological types had similar frequencies of methylation of each of the following genes; *RASSF1A*, *HIN-1*, *RARβ*, *CCND2*, *TWIST* [46]; *RARβ2*, *RASSF1A*, *CCND2* [59]. The study by Fackler et al. [46], however, showed that the same panel of genes had variable sensitivity for the different tumor types: 69% for LCIS, while having a much higher sensitivity in ILC, DCIS, and IDS (100, 95, and 100%, respectively).

A later study carried out by Bae et al. [65] included 60 ductal, 30 lobular, and 19 mucinous invasive breast carcinomas and 8 normal tissue samples obtained from reduction mammoplasty. Using a panel of 12 genes, the authors found that all invasive tumors had at least three genes with

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methylated promoters. They also found that, compared to IDC, mucinous and lobular cancers had a significantly higher mean frequency of methylation. However, the distribution of methylation frequency and number of genes methylated per case showed a significant degree of overlap among diagnostic subgroups. Nevertheless, the high frequency of promoter methylation seen in ILC supports a potential role for promoter methylation analysis in improving the sensitivity of lobular carcinoma detection.

Detection of promoter methylation in circulating DNA

Promoter hypermethylation has also been detected in the serum/plasma of breast cancer cases (Table 2) [66–69]. The results in tumor and blood samples from the same patients show good concordance (Table 3, mean overall concordance is 84%) [53, 70–73]. In a study conducted by Hoque et al. [66], an analysis by disease stage showed that an accumulation of methylation occurs as the disease progresses. This study included a panel of four genes (*APC*, *GSTP1*, *RASSF1A*, and *RARβ2*). Thirty-three percent of stage I/II (8 of 24 patients) and 65% of stage III/IV (43 of 66 patients) plasma samples showed methylation of at least one gene ($p = 0.007$). In a study of 34 women with breast cancer, including 8 with BBD and 20 controls, Dulaimi et al. [53] found that methylation was detectable in the serum of patients with early invasive and pre-invasive disease, while not detectable in normal serum samples, showing specificity of the markers.

Table 3 shows that, in general, the sensitivity of a given gene panel in circulating DNA is slightly lower than the same panel in DNA obtained directly from the tumor. Like studies of tumor DNA, studies of serum DNA have included a variety of gene panels, with some overlap between studies. These panels have shown variable sensitivity with one 4 gene panel (*GSTP1*, *RARβ2*, *RASSF1A*, and *APC*) having a sensitivity of 62% [66], while a different 4 gene panel (*RUNX3*, *p16*, *RASSF1A*, *CDH1*) had a greater sensitivity of 79% [69]. This indicates the importance of gene selection in the sensitivity of the assay.

Because blood collection is a minimally invasive procedure, these studies tend to include more controls than their tissue based counterparts, but the numbers of controls used in each study is still low. The study including the greatest number of control samples ($n = 38$) [66] found low levels of methylation for 2 out of the 4 genes investigated (*RASSF1A*, 5% and *RARβ2*, 8%). The significance of this methylation is unclear and requires further investigation.

The studies reviewed here have used highly variable amounts of DNA though often the precise amount used in the analysis was not reported. Many studies report only the volume of DNA used, rather than the concentration [66–68]. Still others report a range or maximum amount of DNA used in each assay, in these studies 0.05–2 μg [53, 70–72]. Reporting of the amount of DNA used in each assay and the volume of serum/plasma the DNA was obtained from will aid in the design of further studies using serum/plasma samples and clarify the minimum amount of DNA required for successful detection of aberrant changes in methylation patterns.

Limitations of previous studies and need for further research

Pepe [74] and others [75] have suggested steps for the evaluation of new diagnostic markers. Each phase of the evaluation has its own study design and statistical measures [e.g., true-positive rate (TPR) and false-positive rate (FPR)] to assess the usefulness of the assay. Evaluation begins with the identification of potential markers using convenience samples. The next step is to conduct population-based case–control studies testing whether the marker are able to detect established disease. An important aspect of these studies is to use controls arising from the same population as the cases. Studies conducted to date, however, have included either no controls or only "convenience" controls that may not be comparable to the cases with respect to other characteristics. Further, the recommended sample size for adequate precision in calculating a true-positive rate of 0.80 with a standard error of 0.05 and a false-positive of 0.01 as no greater than 0.03 is 110 subjects without cancer and 70 subjects with cancer [75]. Table 1 shows that many studies did not meet these criteria. These studies also did not account for potential confounders, such as age, in the analysis.

Another consequence of the small number of controls included in studies conducted to date is that our knowledge of normal patterns of promoter methylation is limited. A study including fine-needle aspiration biopsies from 55 unaffected women detected promoter methylation of *RARβ2* (9%), *APC* (26%), *H-cadherin* (17%), and *RASSF1A* (37%) [58]. Lewis et al. [58] also showed that methylation frequency increased with risk, as calculated by the Gail model. A recent study of 109 asymptomatic high-risk women, found frequent methylation of *RARβ* (70%), *p16* (29%), *HIN-1* (21%), and *PRA* (77%) and that this was associated with abnormal Masood cytology [76]. Thus, promoter methylation may not be specific to cancer per se, but rather part of an accumulation of changes in DNA that occur over the course of a lifetime, eventually contributing to tumor development. Thus, when using 'normal' tissue samples, it is important to consider the source of the 'normal' tissue, e.g., 'normal' tissue proximal to the tumor, 'normal' tissue from reduction mammoplasty. This will

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Table 2 Characteristics of selected studies of promoter methylation detected in circulation

Reference	Sample type and size	Genes	Methylation frequency (%)	Coverage[a]
Hoque et al. [66][b]	Plasma	GSTP1	26	62%
	Cases	RARβ2	26	
	($n = 47$)	RASSF1A	32	
		APC	17%	
	Plasma	GSTP1	0	
	Healthy controls	RARβ2	8	
	($n = 38$)	RASSF1A	5	
		APC	0	
Müller et al. [67][b]	Serum	ESR1	27	Not calculated
	Cases	APC	23	
	Primary tumors	HSD17β4	12	
	($n = 26$)	HIC1	39	
		RASSF1A	23	
	Recurrent breast cancers	ESR1	70	
	$n = 10$	APC	80	
		HSD17β4	30	
		HIC1	90	
		RASSF1A	80	
	Healthy controls	ESR1	0	
	($n = 10$)	APC	0	
		HSD17β4	0	
		HIC1	10	
		RASSF1A	10	
Papadopoulou et al. [68][b]	Plasma	RASSF1A	26	36%
	Cases	ATM	14	
	($n = 50$)			
	Healthy controls	RASSF1A	0	
	($n = 14$)	ATM	0	
	($n = 9$)			
Tan et al. [69][c]	Serum	RUNX3	47	79%
	Cases	p16	37	
	Metastatic	RASSF1A	42	
	($n = 19$)	CDH1	0	
	Control	All genes	0	
	($n = 10$)			

[a] Coverage, percentage of cases having methylation of at least one gene in the given gene panel (i.e., coverage of 100% means that all samples had methylation of at least one gene in the study's panel)

[b] Methylation analysis was conducted using quantitative real-time methylation-specific PCR (QMSP)

[c] Methylation analysis was conducted using methylation-specific PCR (MSP)

help insure that meaningful case–control comparisons are being conducted.

Promoter hypermethylation has been identified as a potential marker and been shown to be able to detect established breast cancer. Following the path described by Pepe [74], the next step in the evaluation of promoter methylation is to conduct case–control studies nested within prospective cohorts to determine how well it is able to detect pre-clinical disease. Such prospective studies are expensive, require large sample sizes and long follow-up for a sufficient number of cases to be observed. Biological samples collected prospectively from cases are also very valuable and only small sample volumes are usually made available to study any given hypothesis. In light of these considerations we review the questions that remain to be addressed with regard to the evaluation of the potential of methylation analysis for breast cancer screening in the following section.

Need for standardization of methods for methylation analysis

Though a number of studies have been conducted in subjects with established breast cancer, methylation frequencies of genes measured in different labs and in different

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Table 3 Promoter methylation concordance between paired tissue and circulating samples

Reference	Sample size	Genes	Methylation frequency tissue/blood[b] (%)	Coverage[a] tissue/blood (%)	Concordance (%)
Dulaimi et al. (53)	Cases ($n = 34$)	APC	47/29	94/76	81
		RASSF1A	65/56		
		DAPK	50/35		
	Controls ($n = 20$)	All genes	0		
	BBD ($n = 8$)				
Sharma et al. (70)	Cases (IDC) ($n = 36$)	P16	44/36	86/83	89
		P14	47/36		
		CCND2	27/25		
		SLIT2	58/58		
	Controls ($n = 4$)	All genes	0		
Mirza et al. (71)	Cases (IDC) ($n = 50$)	TMS1	24/24	72/64	88
		BRCA1	26/22		
		ESR1	66/48		
		PRB	64/46		
	Controls ($n = 5$)	All genes	0		
Shukla et al. (72)	Cases (IDC) ($n = 20$)	RASSF1A	85/75	85/75	79
		RARβ	10/0		
Hu et al. (73)	Cases (IDC) ($n = 36$)	p16	11/8	31/25	82
		CDH1	25/20		

[a] Coverage, percentage of cases having methylation of at least one gene in the given gene panel (i.e., coverage of 100% means that all samples had methylation of at least one gene in the study's panel); Concordance, between paired samples

[b] In all cases methylation analysis was conducted using methylation-specific PCR (MSP)

sample types have been variable and often not reproducible. This is largely due to 4 factors: (1) variable methods of methylation analysis are used in different studies, (2) gene panels are not consistent across studies, (3) if the same genes are used, often different promoter CpG sites are used, and (4) sources of DNA are variable from study to study (i.e., serum, plasma, tissue, biopsy).

The first issue to be addressed is the selection of the optimal method for methylation analysis. Optimization for small sample volumes (and therefore a small amount of DNA template) should be the focus, to allow for the use of samples obtained from existing prospective studies which are needed to assess the ability of promoter methylation patterns to detect pre-clinical disease. This will require the determination of the absolute sensitivity of the different methods. The absolute sensitivity of an assay is the minimum quantity of target DNA required for successful amplification and detection [77]. To accomplish this, it is suggested here that criteria for publication of methylation data be standardized and include the requirement for confirmation of methylation results from non-sequence based methods (i.e., MSP and QMSP) by bisulfite sequencing (the gold standard) for a subset of samples. It is also suggested that the amount of DNA used in each assay and the coefficients of variation (CV) for any repeat measures, be reported.

Reproducibility of methylation results is an area of great importance, one that has not been sufficiently addressed in the current literature. Methylation frequencies have largely not been reproducible across studies. This variability may be reduced with the standardization of methods and reporting of results. One study designed to specifically examine the reproducibility of the PMR (percent of fully methylated DNA found in a sample), was based on QMSP analysis of DNA from paraffin-embedded colon cancer samples. This study found the PMR to have high inter-assay CVs with an average of 21% (range 10–38%) [78]. In a recent study, methylation results using a nested QMSP method (QAMA) on DNA obtained from microdissected cells from formalin-fixed and paraffin-embedded tumor tissues ($n = 13$) was found to have a good correlation with sequencing results ($R = 0.982$). To our knowledge, no studies have reported the reproducibility of measurements obtained from serum or plasma samples.

Because no single gene has been found to be methylated in all breast cancers, it is necessary to use a panel of genes.

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The variability in the genes included in each panel makes it difficult to compare or combine the results of different studies and to infer how promoter methylation would fare as a screening tool. Further, though two studies may have included the same genes in their panels, they have not necessarily probed for the same CpG sites within the promoter. This adds another layer of variability between studies and there is no consensus or criteria for the selection of CpG sites within a promoter.

Regarding panel selection, up to now, it has been based largely on the candidate gene approach, using genes that have a known involvement in carcinogenesis. Methylation of these genes can be found in many other forms of cancer and is not specific to breast cancer. There are genes, however, that may have an increased role in breast cancer specifically, such as *GSTP1*; which is known to be involved in hormone related cancers [79], *BRCA1*; a known player in the family history of breast cancer such that patients with methylated *BRCA1* having a similar phenotype to those with *BRCA1* mutations [80], and *ERS1* and its associated genes because of the known role of estrogen in breast carcinogenesis [81]. The inclusion of these genes may help improve the specificity of a gene panel for breast cancer.

It will also be important to understand those factors that influence methylation analysis, such as the source of DNA (i.e., serum vs. plasma), sample volume, sample handling, storage temperature, and duration and freeze/thaw cycles. For example, variation in the amount of DNA obtained from serum and plasma has been shown. The major difference between serum and plasma is the presence of clotting factors (and associated proteins) in plasma. It appears that serum tends to contain approximately sixfold more DNA than plasma does. Much of this DNA, however, could come from the normal DNA of contaminating leukocytes [82]. Further, large prospective studies needed to test the diagnostic potential of these markers requires long periods of sample storage as cases are accrued through follow-up. The effect of this long-term storage on DNA methylation also needs examining. The Early Detection Research Network (EDRN) of the National Cancer Institute (NCI) has made understanding those factors that may influence methylation analysis, part of their focus [83].

Summary and conclusion

Localized breast cancer has a 5-year survival rate of 98%. However, when diagnosed after the tumor has metastasized, the survival rate decreases drastically to 27% [1]. These results point to the benefit of screening and early detection. Given that mammography sensitivity is as low as 50% in some groups of women, the potential for methylation markers in circulating DNA, to complement the results of mammography in breast cancer detection and diagnosis, deserves further exploration [84] and has been the focus of this review.

Changes in promoter methylation status are frequent events that occur early in the tumorigenic process and are detectable through minimally invasive measures. A number of cancer-related genes have been found to be frequently methylated in breast cancer. These markers show promise for distinguishing between malignant disease and benign disease or normal tissue, and they may be able to improve the detection of lobular carcinomas. Furthermore, the combination of this minimally invasive procedure with mammography could improve the sensitivity of tumor detection in women with high breast density, a characteristic that is associated with an increased breast cancer risk [85] and reduced sensitivity of mammography [10–12].

Additional questions of interest include whether methylation patterns vary with ER/PR status [81, 86, 87], and in *BRCA* mutation carriers or in familial breast cancers [76, 88]. Ideally, to be a successful screening tool, a marker would be able to detect breast cancer, regardless of its receptor status, origin or subtype. The selection of the genes to form the diagnostic panel will likely determine how successful promoter methylation is in identifying breast cancer and the type of breast cancer it is detecting.

Currently, most studies select genes based on known gene function and methylation frequency. As we gain a better understanding of the methylome, a map of genome-wide, tissue-specific patterns of methylation [89] is expected to change. Microarrays designed specifically for bisulfite-treated DNA are available but currently are not optimized for high-throughput analysis and account for only 0.1% of the total CpG sites in the human genome [89]. The future of methylation analysis will likely involve a combination of isolation of the methylated fraction of DNA either using MBD proteins [Methylated-CpG Island Recovery Assay (MIRA)] or immunocapture [Methylated DNA Immunoprecipitation on Chips (MeDIP-chip)] methods and next generation microarray or sequencing technologies [89]. The optimal method for analysis, however, will ultimately depend on the research goals of the analysis since currently no one method is able to balance the need for quantitative accuracy, sensitive detection, local versus global information, and automation [90].

Studies in well-defined populations, including appropriate controls and larger numbers are needed to further evaluate the potential of DNA methylation to improve current breast cancer screening strategies. In order to successfully conduct these studies, optimization and standardization of methylation detection assays that can be used on small volumes of serum/plasma frozen for extended periods of time are needed.

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